PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12Q 1/68, C07K 14/22, 14/245, C12R 1/19, 1/36, C12N 15/10, C12Q 1/18

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(22) International Filing Date: 23 September 1999 (23.09.99)

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60/105,965 28 October 1998 (28.10.98) US

(71) Applicant *(for all designated States except US)*: WARNER-LAMBERT COMPANY [US/US]; 201 Tabor Road, Morris Plains, NJ 07950 (US).

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(75) Inventors/Applicants *(for US only)*: DUNHAM, Steven, Alan [US/US]; 1600 Northbrook Drive, Ann Arbor, MI 48103 (US). OLSON, Eric [US/US]; 534 Glendale Circle, Ann Arbor, MI 48103 (US).

(74) Agents: RYAN, M., Andrea; Warner-Lambert Company, 201 Tabor Road, Morris Plains, NJ 07950 (US) et al.

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, TZ, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: METHODS OF IDENTIFYING AND CHARACTERIZING MUTATIONS WITHIN BACTERIAL DNA GYRASE AND FABI

(57) Abstract

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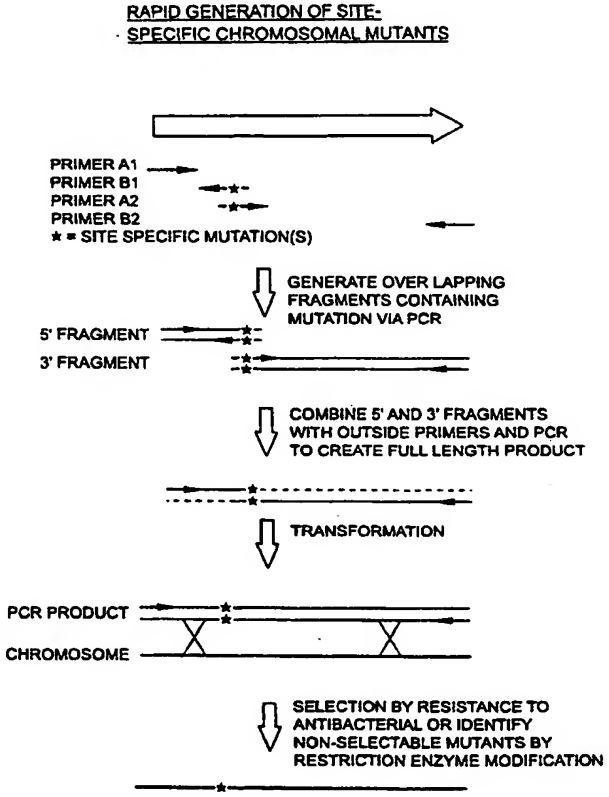

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MCMURRY L M ET AL: "Triclosan targets lipid synthesis" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 394, no. 394, 6 August 1998 (1998-08-06), pages 531-532-532, XP002108567 ISSN: 0028-0836 cited in the application the whole document	5,6,8,9, 14,15, 20-22, 24,36
A	DEGUCHI T ET AL.: "DNA gyrase mutations in quinolone-resistant clinical isolates of Neisseria gonorrhoeae" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 39, no. 2, 1995, pages 561-563, XP000870151 cited in the application the whole document	
A	WEIGEL L ET AL: "GyrA mutations associated with fluoroquinolone resistance in eight species of enterobacteriaceae" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, vol. 42, no. 10, October 1998 (1998-10), pages 2661-2667-67, XP002118443 ISSN: 0066-4804 the whole document	
A	EP 0 688 873 A (BAYER AG) 27 December 1995 (1995-12-27) the whole document	
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P,Y	* see especially page 30318, column 2, paragraph 2 * the whole document	5,6,9, 13-15, 20,35

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METHODS OF IDENTIFYING AND CHARACTERIZING MUTATIONS WITHIN BACTERIAL DNA GYRASE AND FABI

BACKGROUND OF THE INVENTION

Discovery and development of an antibacterial agent is aided by the knowledge by which the compound inhibits cell growth. A common technique in elucidating this information is to isolate mutants that alter the susceptibility of the organism to the compound and identify what mutation is responsible for the phenotype. By identifying what gene the mutation lies in, or affects the expression of, one can often learn what cellular pathway the compound inhibits, what the compounds binds to to affect growth, and obtain information about how the compound binds to the target molecule.

Random mutagenesis followed by phenotypic selection, such as resistance to an antibacterial agent, has been shown to be an effective technique to establish structure-function relationships for proteins in yeasts, viruses and bacteria (Kwan T., Gros P., *Biochemistry*, 1998;37:3337-3350; Loeb D.D. et al., *Nature*, 1989;340(6232):397-400; Rolli V. et al., *Biochemistry*, 1997;36:12147-12154). The success of such experiments is determined, in part, by the randomness of the mutagenic procedure, the ability to select for mutants of interest, the numbers of mutant cells that can be generated, and the ability to identify the mutation that is responsible for the phenotype of a selected mutant. This procedure has been used to isolate mutants of genes that are cloned into plasmids or other extrachromosomal elements. While this can work in some instances, the technique is labor intensive and is complicated in cases where the strain is diploid for the gene of interest or toxic when the gene of interest is expressed from a multicopy plasmid.

Recently, Kok et al have shown that combining mutagenesis of a defined fragment by PCR with natural transformation is a way to identify mutations which abolish the function of PobR in *Acinetobacter* region (Kok R., D'Argenio D., and Ornston L.N., Combining localized PCR mutagenesis and natural transformation in direct genetic analysis of a transcriptional regulator gene, *pobR*. *J. Bac.*, 1997;179:4270-4276). This technique exploits the ability of *Acinetobacter* to take

INTERNA. ONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 99/22118

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5686590 A	11-11-1997	AU 690121 B	23-04-1998
		AU 6912194 A	12-12-1994
		AU 6949694 A	12-12-1994
		CA 2162868 A	24-11-1994
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		WO 9426312 A	24-11-1994
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		CA 2152218 A	24-12-1995
		HU 71861 A	28-02-1996
		JP 8000298 A	09-01-1996

resistance in spontaneous mutants and mutants generated using DNA damaging agents.

SUMMARY OF THE INVENTION

This instant invention is a method for identifying molecular targets in bacteria treated with an antibacterial compound. The method is based on creating and identifying mutations in bacteria that confer altered susceptibility to an antibacterial compound. The mutations provide valuable information about the molecular target of the compound and how the compound and target interact. The bacterial strains generated can be used to provide information that could be useful in identifying and characterizing compounds that could be used or developed for treating bacterial infections of humans, other animals and plants.

Using *Neisseria gonorrhoeae*, we subjected *gyrA* or *fabI* to site-specific and random nucleotide mutagenesis to identify mutations that conferred resistance to ciprofloxacin or diphenyl ethers, respectively. These experiments identified previously described and novel mutations associated with resistance to these compounds. These experiments also demonstrate the ability to create and identify mutations in *Neisseria gonorrhoeae* associated with resistance to antibacterial compounds by combining random mutagenesis with phenotypic selection.

The instant invention is a system that allows for the simultaneous creation and identification of mutations that confer resistance to antibacterial compounds.

This technology is for the identification, or isolation and identification, of mutations responsible for altered susceptibility of several bacteria to chemicals (or any other selectable phenotype). This invention can be used in any bacteria that can be transformed with DNA, can carryout homologous recombination and for which the genome sequence can be determined. Examples of these include, but are not limited to: *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Acinetobacter*, *Escherichia coli*, *Staphylococcus aureus*,

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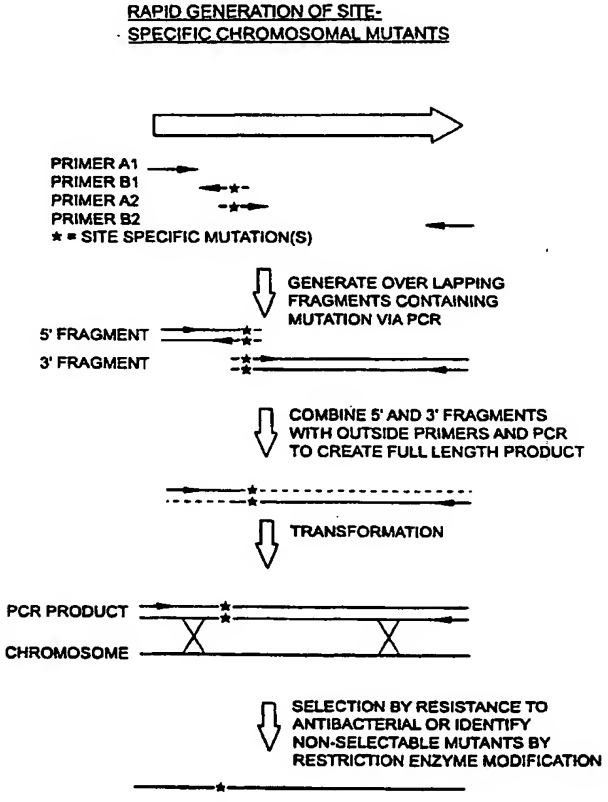

kilobasepairs for each PCR product) and use to amplify chromosomal DNA from the strain isolated in 'd'.

f) Transforming N400 with the PCR products from 'e' to define the approximately 2 kilobasepair or smaller region of the chromosome that has the mutation or mutations responsible for the altered susceptibility;

g) Sequencing the DNA from the approximately 2 kilobasepair or smaller region defined in 'f'.

h) Comparing the DNA sequence with DNA sequence from the same region from N400. If a single change in the order of the nucleotides is found, this change is defined as a mutation which confers altered susceptibility to the compound. If more than one change is observed, additional rounds of primer design, PCR amplification, transformation and selections are executed so that the contribution of each mutation to the phenotype can be determined.

This invention can also be used to identify mutations that confer altered susceptibility to a chemical in strains of *Neisseria gonorrhoeae* that have previously been isolated using other methods. In this case, Step 'a' above would be:

a) Generating a defined set of overlapping PCR products (about 10 kilobasepairs per product) using chromosomal DNA from a mutant strain of *Neisseria gonorrhoeae* as a template that had previously been generated and demonstrated to be more or less susceptible to a chemical than N400. The PCR products, taken together, comprise the complete DNA composition of the chromosome of the mutant organism.

Steps b-h would be identical to that described above.

Further, the invention is a process for identifying and characterizing drug-target interactions using *Neisseria gonorrhoeae* comprising:

a) mutagenizing randomly a defined region of the chromosome that may alter susceptibility to chemical compounds. This region can

METHODS OF IDENTIFYING AND CHARACTERIZING MUTATIONS WITHIN BACTERIAL DNA GYRASE AND FABI

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(a) generating DNA fragments by polymerase chain reaction amplification of the bacterial chromosome corresponding to regions of the bacterial chromosome which may contain a mutation;

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(c) isolating bacteria that demonstrate the identifiable phenotype; and

repeating steps a through c until a single DNA fragment less than about 10 kilobases in length is identified as being responsible for the mutation; and

identifying the mutation contained in the DNA fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the generation of site-specific mutants using splicing by overlapping extension.

Figure 2 shows the generation of *Neisseria gonorrhoeae* with quinolone resistant mutations in *gyrA*.

Figure 3 shows transformation efficiencies and genotypes isolated from PCR-mediated mutagenesis of *gyrA*.

Figure 4 illustrates an overview of rapid antimicrobial target elucidation.

DETAILED DESCRIPTION OF THE INVENTION

Understanding the mechanism of inhibition of antibacterial compounds is beneficial to the discovery and development of an effective antibiotic. Natural-competence and the highly recombinant nature of *Neisseria gonorrhoeae* make this organism ideal for identifying and characterizing drug-target interactions. We use *Neisseria gonorrhoeae* to demonstrate the utility of this invention, however

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shown in Table 1. Strains generated as a result of these experiments are NG-2691, NG-2698, GC 156 and GC 158.

TABLE 1. *gyrA* Ciprofloxacin Resistant Mutations Identified by QRDR Random Mutagenesis with a Degenerate Oligonucleotide

Quinolone resistant genotypes																
H	G	D	S	A	V	Y	D	T	I	V	R	M	A	Q	N	(Seq.1)
		E	F				G									
			C				N									
							H									
							E									
							A									

To identify other mutations leading to ciprofloxacin resistance that may not be located between residues 88 and 103 of gyrA, PCR of an 8.8 kilobasepair fragment containing *gyrA* was performed to create a pool of PCR products with random nucleotide substitutions distributed across the entire region (Kok R. et al, 1997). This pool was subsequently introduced into N400 by transformation and strains with mutations leading to ciprofloxacin resistance were isolated (Figure 3). Ciprofloxacin resistant colonies were observed at a frequency of 10^{-2} for bacteria transformed with the PCR generated library. This frequency was at least 4 orders of magnitude higher than that observed for cells that were not transformed indicating that the mutations were likely generated as a result of the PCR amplification and in the region of chromosome corresponding to the 8.8 kilobasepair PCR product used in the transformation.

To identify where in the 8.8 kilobasepair fragment the mutation responsible for the resistance was located, oligonucleotide primer pairs were designed to PCR amplify an 800 base pair product of the 5' portion of *gyrA* containing the QRDR. Each PCR product was then used to transform ciprofloxacin sensitive strains and, in all cases, was able to generate ciprofloxacin resistant colonies at high frequencies. Therefore, all ciprofloxacin resistant strains generated using an 8.8 kilobasepair random library contained a mutation in the 800 base pair region containing the 5' region of *gyrA*.

kilobasepairs for each PCR product) and use to amplify chromosomal DNA from the strain isolated in 'd'.

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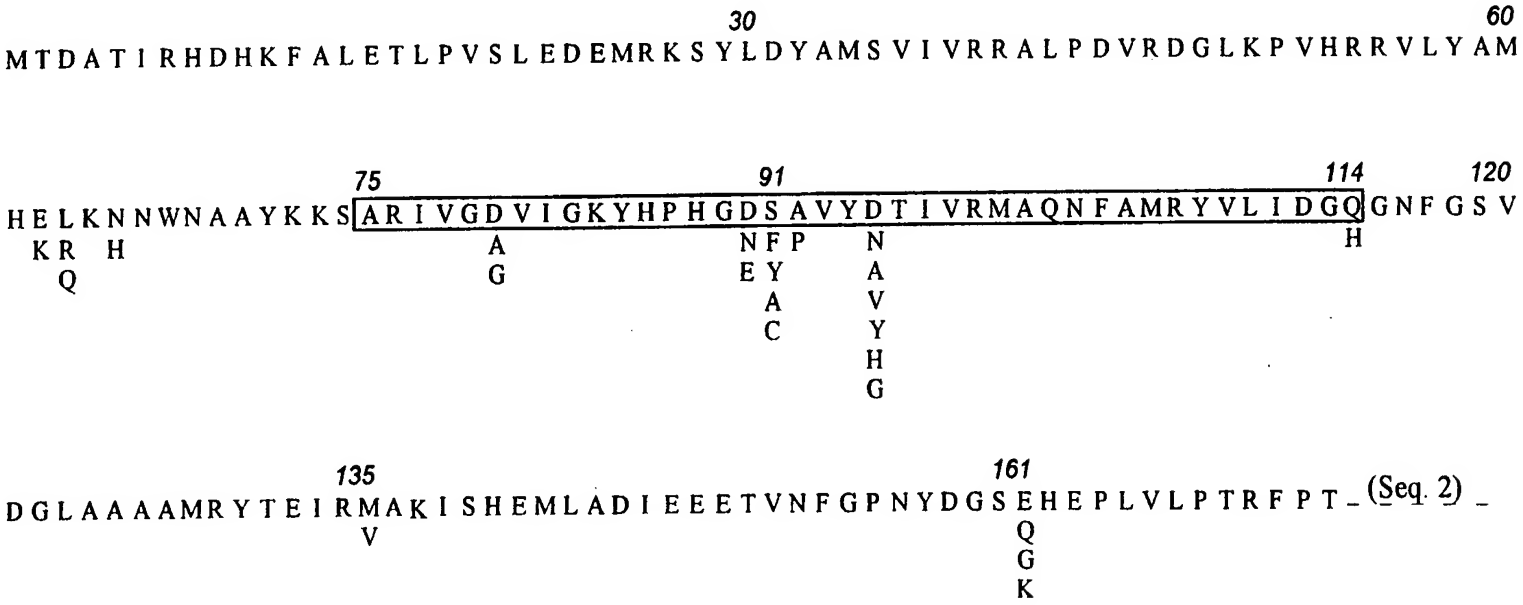
Amino Acid Sequence of N400 GyrA and Quinolone Resistant Mutants



(Seq. 2)

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Table 2: *Neisseria gonorrhoeae* strains

Strain Name	Parent	Genotype	Codon alteration	Phenotype for selection
NG-2707	N400	*gyrA* E62K	GAG-AAG	Clinafloxacin resistant
GC318	N400	*gyrA* L63Q	CTG-CAG	Clinafloxacin resistant
NG-2721	N400	*gyrA* L63R	CTG-CGG	Clinafloxacin resistant
NG-2711	N400	*gyrA* N65H	AAT-CAT	Clinafloxacin resistant
NG-2706	N400	*gyrA* D80G	GAC-GGC	Clinafloxacin resistant
NG-2717	N400	*gyrA* D80A	GAC-GCC	Clinafloxacin resistant
NG-2687	N400	*gyrA* S91F	TCC-TTC	Clinafloxacin resistant
GC158	N400	*gyrA* S91C	TCC-TGC	Clinafloxacin resistant
NG-2690	N400	*gyrA* S91A	TCC-GCC	Clinafloxacin resistant
GC219	N400	*gyrA* A92P	GCA-TCA	Clinafloxacin resistant
GC291	N400	*gyrA* D95G	GAC-GGC	Clinafloxacin resistant
NG-2691	N400	*gyrA* D95A	GAC-GCC	Clinafloxacin resistant
NG-2720	N400	*gyrA* D95V	GAC-GGC	Clinafloxacin resistant
NG-2723	N400	*gyrA* D95Y	GAC-TAC	Clinafloxacin resistant
GC156	N400	*gyrA* D95E	GAC-GAG	Clinafloxacin resistant
NG-2698	N400	*gyrA* D95H	GAC-CAC	Clinafloxacin resistant
NG-2709	N400	*gyrA* S91F,D95G	TCC-TTC, GAC-GGC	Clinafloxacin resistant
NG-2716	N400	*gyrA* Q114H	CAG-CAT	Clinafloxacin resistant
NG-2719	N400	*gyrA* M135V	ATG-GTG	Clinafloxacin resistant
NG-2712	N400	*gyrA* E161G	GAA-GGA	Clinafloxacin resistant
NG-2669	N400	*fabI* I15V	ATT-GTT	DHDPE resistant
NG-2654	N400	*fabI* I20T	ATC-ACC	DHDPE resistant
NG-2651	N400	*fabI* G23S	GGC-AGC	DHDPE resistant
NG-2670	N400	*fabI* A25V	GCC-GTC	DHDPE resistant
NG-2660	N400	*fabI* M51T	ATG-ACG	DHDPE resistant
NG-2641	N400	*fabI* S91T	TCC-ATC	DHDPE resistant
NG-2639	N400	*fabI* D86D, G93A	GAC-GAT, GGC-GCG	DHDPE resistant
NG-2638	N400	*fabI* G93S	GGC-AGC	DHDPE resistant
NG-2640	N400	*fabI* G93C	GGC-TGC	DHDPE resistant
NG-2648	N400	*fabI* G93V	GGC-GTC	DHDPE resistant
NG-2657	N400	*fabI* A95T	GCG-ACG	DHDPE resistant
NG-2656	N400	*fabI* A99G	GCC-GGC	DHDPE resistant
NG-2653	N400	*fabI* F104V	TTC-GTC	DHDPE resistant
NG-2658	N400	*fabI* L105H	CTC-CAC	DHDPE resistant
NG-2663	N400	*fabI* A144V	GCC-GTC	DHDPE resistant
NG-2642	N400	*fabI* Y147H	TAC-CAC	DHDPE resistant
NG-2671	N400	*fabI* G149A	GGC-GCC	DHDPE resistant
NG-2652	N400	*fabI* V159A	GTG-GCG	DHDPE resistant
NG-2661	N400	*fabI* M160I	ATG-ATA	DHDPE resistant
NG-2644	N400	*fabI* M162V	ATG-GTG	DHDPE resistant
NG-2667	N400	*fabI* I193V, Q5Q	CAA-CAG, ATC-GTC	DHDPE resistant
NG-2665	N400	*fabI* I193N	ATC-AAC	DHDPE resistant
NG-2655	N400	*fabI* T195S	ACG-TCG	DHDPE resistant
NG-2643	N400	*fabI* I201V	ATC-GTC	DHDPE resistant
NG-2666	N400	*fabI* D203V	GAT-GTT	DHDPE resistant
NG-2664	N400	*fabI* D203Y	GAT-TAT	DHDPE resistant
NG-2647	N400	*fabI* F204A	TTC-GCG	DHDPE resistant
NG-2646	N400	*fabI* F204L	TTC-TTG	DHDPE resistant
NG-2650	N400	*fabI* F204S	TTC-TCC	DHDPE resistant
NG-2649	N400	*fabI* F204I	TTC-ATC	DHDPE resistant
NG-2645	N400	*fabI* F204H	TTC-CAC	DHDPE resistant
NG-2659	N400	*fabI* A212T	GCC-ACC	DHDPE resistant
NG-2662	N400	*fabI* A212V	GCC-GTC	DHDPE resistant
NG-2672	N400	*fabI* Y247N	TAT-AAT	Triclosan resistant

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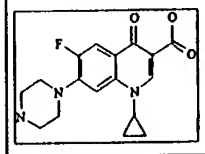
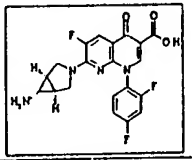
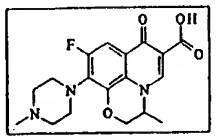
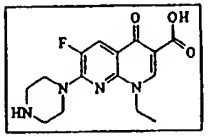
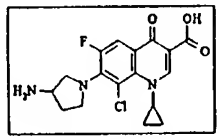
							E									
							A									

To identify other mutations leading to ciprofloxacin resistance that may not be located between residues 88 and 103 of gyrA, PCR of an 8.8 kilobasepair fragment containing *gyrA* was performed to create a pool of PCR products with random nucleotide substitutions distributed across the entire region (Kok R. et al, 1997). This pool was subsequently introduced into N400 by transformation and strains with mutations leading to ciprofloxacin resistance were isolated (Figure 3). Ciprofloxacin resistant colonies were observed at a frequency of 10^{-2} for bacteria transformed with the PCR generated library. This frequency was at least 4 orders of magnitude higher than that observed for cells that were not transformed indicating that the mutations were likely generated as a result of the PCR amplification and in the region of chromosome corresponding to the 8.8 kilobasepair PCR product used in the transformation.

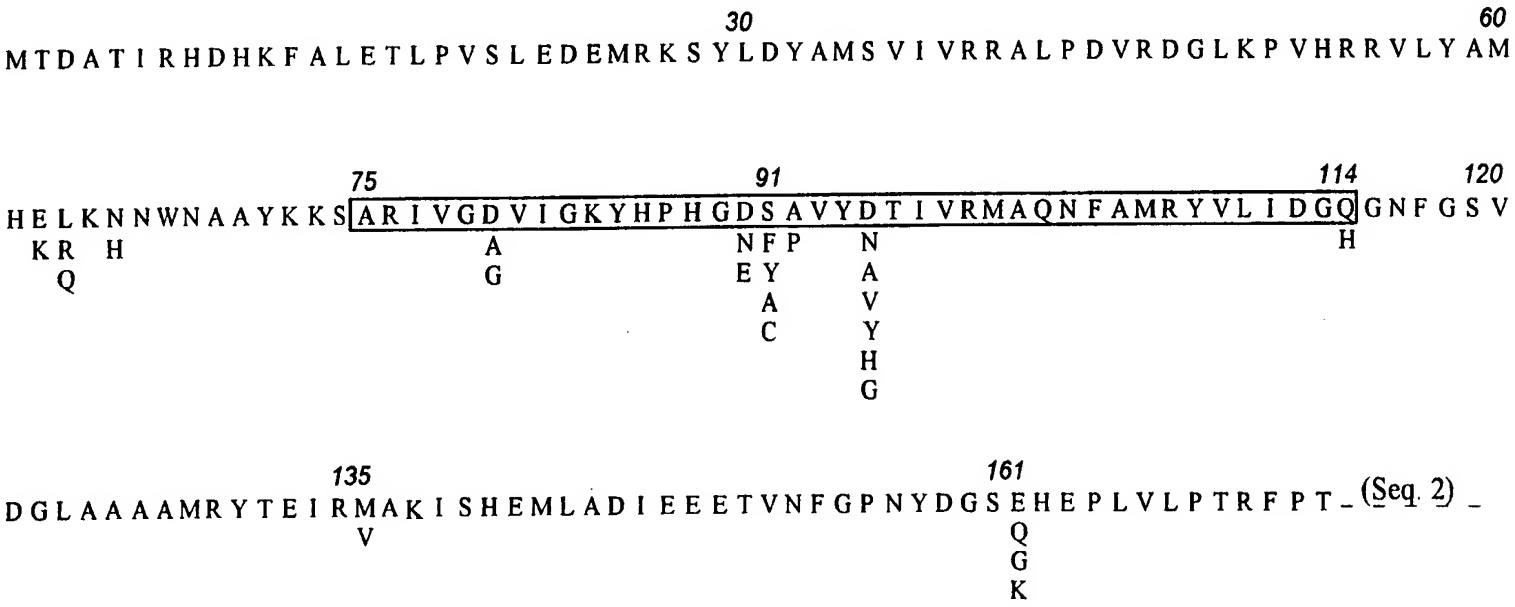
To identify where in the 8.8 kilobasepair fragment the mutation responsible for the resistance was located, oligonucleotide primer pairs were designed to PCR amplify an 800 base pair product of the 5' portion of *gyrA* containing the QRDR. Each PCR product was then used to transform ciprofloxacin sensitive strains and, in all cases, was able to generate ciprofloxacin resistant colonies at high frequencies. Therefore, all ciprofloxacin resistant strains generated using an 8.8 kilobasepair random library contained a mutation in the 800 base pair region containing the 5' region of *gyrA*.

Table 3. Susceptibilities of *N. gonorrhoeae gyrA* and *parC* mutants to a panel of quinolones

			Minimum Inhibitory Concentration [a] (μg/ml)					
			Ciprofloxacin	Trovafloxacin	Ofloxacin	Enoxacin	Clinafloxacin	Erythromycin
								
Strain	*gyrA* genotype	*parC* genotype						
N400	WT	WT	0.002	0.002	0.008	.06-.12	0.001	4
GC8	S91F,D95G	WT	0.06	0.03	0.25	4	0.03	2
GC19	S91F,D95G	S88P,E91K	4	4	8	64	0.25	4
GC23	WT	S88P,E91K	0.002	0.002	0.008	.06-.12	0.001	2
GC10	S91A	WT	0.008	0.008	0.03	1	0.004	2
GC158	S91F	WT	0.03	0.015	0.12	2	0.008	2
GC155	S91C	WT	0.004	0.004	0.015	.25-.5	0.002	4
GC11	D95A	WT	0.008	0.008	0.03	1	0.004	4
GC56	D95G	WT	0.015	0.008	0.06	2	0.008	2
GC59	D95H	WT	0.015	0.008	0.06	2	0.008	2
GC85	V93A	WT	0.002	0.004	0.008	.06-.12	0.002	2-4
GC87	M100A	WT	0.002	.002-.004	0.015	0.12	0.002	4

[a] As determined using microdilution susceptibility tests

Amino Acid Sequence of N400 GyrA and Quinolone Resistant Mutants



(Seq. 2)

Amino Acid Sequence of FabI and DHDPE or Triclosan Resistant Mutations

```
                                                  30                                                  60
MGFLQGKKILITGMISERSIAYGIAKACREQGAELAFTYVVDKLEERVRKMAAELDSELV
                  V       T   S V                                T

                                                  90                                                 120
FRCDVASDDEINQVFADLGKHWDGLDGLVHSIGFAPKEALSGDFLDSISREAFNTAHEIS
                                T CSV V     G   LH

                                                 150                                                 180
AYSLPALAKAARPMMRGRNSAIVALSYLGAVRAIPNYNVMGMAKASLEAGIRFTAACLGK
                              V   H                 AI  V

                                                 210                                                 240
EGIRCNGISAGPIKTLAASGIADFGKLLGHVAAHNPLRRNVTIEEVGNTAAFLLSDLSSG
                    NS      V YV SLIV          TV

                                                261
ITGEITYVDGGYSINALSTEG
                  N
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(Seq. 3)

Table 2: *Neisseria gonorrhoeae* strains

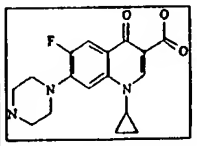
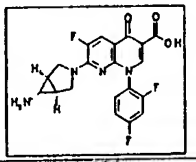
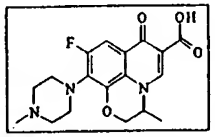
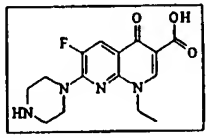
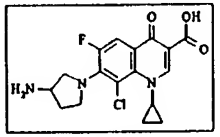
Strain Name	Parent	Genotype	Codon alteration	Phenotype for selection
NG-2707	N400	*gyrA* E62K	GAG-AAG	Clinafloxacin resistant
GC318	N400	*gyrA* L63Q	CTG-CAG	Clinafloxacin resistant
NG-2721	N400	*gyrA* L63R	CTG-CGG	Clinafloxacin resistant
NG-2711	N400	*gyrA* N65H	AAT-CAT	Clinafloxacin resistant
NG-2706	N400	*gyrA* D80G	GAC-GGC	Clinafloxacin resistant
NG-2717	N400	*gyrA* D80A	GAC-GCC	Clinafloxacin resistant
NG-2687	N400	*gyrA* S91F	TCC-TTC	Clinafloxacin resistant
GC158	N400	*gyrA* S91C	TCC-TGC	Clinafloxacin resistant
NG-2690	N400	*gyrA* S91A	TCC-GCC	Clinafloxacin resistant
GC219	N400	*gyrA* A92P	GCA-TCA	Clinafloxacin resistant
GC291	N400	*gyrA* D95G	GAC-GGC	Clinafloxacin resistant
NG-2691	N400	*gyrA* D95A	GAC-GCC	Clinafloxacin resistant
NG-2720	N400	*gyrA* D95V	GAC-GGC	Clinafloxacin resistant
NG-2723	N400	*gyrA* D95Y	GAC-TAC	Clinafloxacin resistant
GC156	N400	*gyrA* D95E	GAC-GAG	Clinafloxacin resistant
NG-2698	N400	*gyrA* D95H	GAC-CAC	Clinafloxacin resistant
NG-2709	N400	*gyrA* S91F,D95G	TCC-TTC, GAC-GGC	Clinafloxacin resistant
NG-2716	N400	*gyrA* Q114H	CAG-CAT	Clinafloxacin resistant
NG-2719	N400	*gyrA* M135V	ATG-GTG	Clinafloxacin resistant
NG-2712	N400	*gyrA* E161G	GAA-GGA	Clinafloxacin resistant
NG-2669	N400	*fabI* I15V	ATT-GTT	DHDPE resistant
NG-2654	N400	*fabI* I20T	ATC-ACC	DHDPE resistant
NG-2651	N400	*fabI* G23S	GGC-AGC	DHDPE resistant
NG-2670	N400	*fabI* A25V	GCC-GTC	DHDPE resistant
NG-2660	N400	*fabI* M51T	ATG-ACG	DHDPE resistant
NG-2641	N400	*fabI* S91T	TCC-ATC	DHDPE resistant
NG-2639	N400	*fabI* D86D, G93A	GAC-GAT, GGC-GCG	DHDPE resistant
NG-2638	N400	*fabI* G93S	GGC-AGC	DHDPE resistant
NG-2640	N400	*fabI* G93C	GGC-TGC	DHDPE resistant
NG-2648	N400	*fabI* G93V	GGC-GTC	DHDPE resistant
NG-2657	N400	*fabI* A95T	GCG-ACG	DHDPE resistant
NG-2656	N400	*fabI* A99G	GCC-GGC	DHDPE resistant
NG-2653	N400	*fabI* F104V	TTC-GTC	DHDPE resistant
NG-2658	N400	*fabI* L105H	CTC-CAC	DHDPE resistant
NG-2663	N400	*fabI* A144V	GCC-GTC	DHDPE resistant
NG-2642	N400	*fabI* Y147H	TAC-CAC	DHDPE resistant
NG-2671	N400	*fabI* G149A	GGC-GCC	DHDPE resistant
NG-2652	N400	*fabI* V159A	GTG-GCG	DHDPE resistant
NG-2661	N400	*fabI* M160I	ATG-ATA	DHDPE resistant
NG-2644	N400	*fabI* M162V	ATG-GTG	DHDPE resistant
NG-2667	N400	*fabI* I193V, Q5Q	CAA-CAG, ATC-GTC	DHDPE resistant
NG-2665	N400	*fabI* I193N	ATC-AAC	DHDPE resistant
NG-2655	N400	*fabI* T195S	ACG-TCG	DHDPE resistant
NG-2643	N400	*fabI* I201V	ATC-GTC	DHDPE resistant
NG-2666	N400	*fabI* D203V	GAT-GTT	DHDPE resistant
NG-2664	N400	*fabI* D203Y	GAT-TAT	DHDPE resistant
NG-2647	N400	*fabI* F204A	TTC-GCG	DHDPE resistant
NG-2646	N400	*fabI* F204L	TTC-TTG	DHDPE resistant
NG-2650	N400	*fabI* F204S	TTC-TCC	DHDPE resistant
NG-2649	N400	*fabI* F204I	TTC-ATC	DHDPE resistant
NG-2645	N400	*fabI* F204H	TTC-CAC	DHDPE resistant
NG-2659	N400	*fabI* A212T	GCC-ACC	DHDPE resistant
NG-2662	N400	*fabI* A212V	GCC-GTC	DHDPE resistant
NG-2672	N400	*fabI* Y247N	TAT-AAT	Triclosan resistant

generate the library of DNA fragments used in the transformation that created mutant strain. Once the mutation has been mapped to a reasonable sized portion of the chromosome, for example less than 3 kilobasepairs, using an iterative process of primer design, PCR amplification, transformation and selection of bacteria with altered susceptibility to the chemical, the DNA from the region of the mutant that carries the mutation can be sequenced. In this manner the mutation responsible for the altered susceptibility can be identified. From this the gene or genes involved in the mechanism by which the chemical affects the growth of the bacteria are identified.

This system can also be used to identify mutations in a bacterial chromosome that have been generated by other means and result in a phenotypic alteration. Examples of this are 1) strains carrying extra-chromosomal elements that result in a detectable phenotype, such as loss of virulence, fluorescence via green fluorescence protein (GFP) or resistance to an antibacterial compound; or 2) mutant strains containing point mutations that result in resistance to antibacterial compounds with known or unknown targets. In the former case, PCR products containing the entire genome can be systematically subjected to in vitro mutagenesis where any external fragment of DNA can be randomly inserted into the PCR product using the GPS system of New England Biolabs. The resulting PCR products can then be transformed into the wild-type strain, the extra-chromosomal material recombined onto the chromosome, and mutants containing the desired phenotype identified and isolated. In the latter case, resistant mutants can be generated using chemical means such as ethylenemethane sulfonate, DNA-damaging agents such as UV irradiation, or simply by isolating spontaneous mutants that grow on plates containing a concentration of the chemical compound that prevents growth of the parent strain. Once a strain carrying the detectable phenotype has been generated, PCR of the entire chromosome of the mutant organism in defined regions can be performed and the location of the mutation identified as described above.

This invention allows one to identify genes and gene products that can be mutated and result in an altered phenotype such as changing an organism's susceptibility to a particular chemical. This can be done without any prior information about where in the chromosome such mutations would have to occur

Table 3. Susceptibilities of *N. gonorrhoeae gyrA* and *parC* mutants to a panel of quinolones

			Minimum Inhibitory Concentration * (μg/ml)					
			Ciprofloxacin	Trovafloxacin	Ofloxacin	Enoxacin	Clinafloxacin	Erythromycin
								
Strain	*gyrA* genotype	*parC* genotype						
N400	WT	WT	0.002	0.002	0.008	.06-.12	0.001	4
GC8	S91F,D95G	WT	0.06	0.03	0.25	4	0.03	2
GC19	S91F,D95G	S88P,E91K	4	4	8	64	0.25	4
GC23	WT	S88P,E91K	0.002	0.002	0.008	.06-.12	0.001	2
GC10	S91A	WT	0.008	0.008	0.03	1	0.004	2
GC158	S91F	WT	0.03	0.015	0.12	2	0.008	2
GC155	S91C	WT	0.004	0.004	0.015	.25-.5	0.002	4
GC11	D95A	WT	0.008	0.008	0.03	1	0.004	4
GC56	D95G	WT	0.015	0.008	0.06	2	0.008	2
GC59	D95H	WT	0.015	0.008	0.06	2	0.008	2
GC85	V93A	WT	0.002	0.004	0.008	.06-.12	0.002	2-4
GC87	M100A	WT	0.002	.002-.004	0.015	0.12	0.002	4

*As determined using microdilution susceptibility tests

Generation of the PCR Product Containing site-specific *gyrA* Mutations

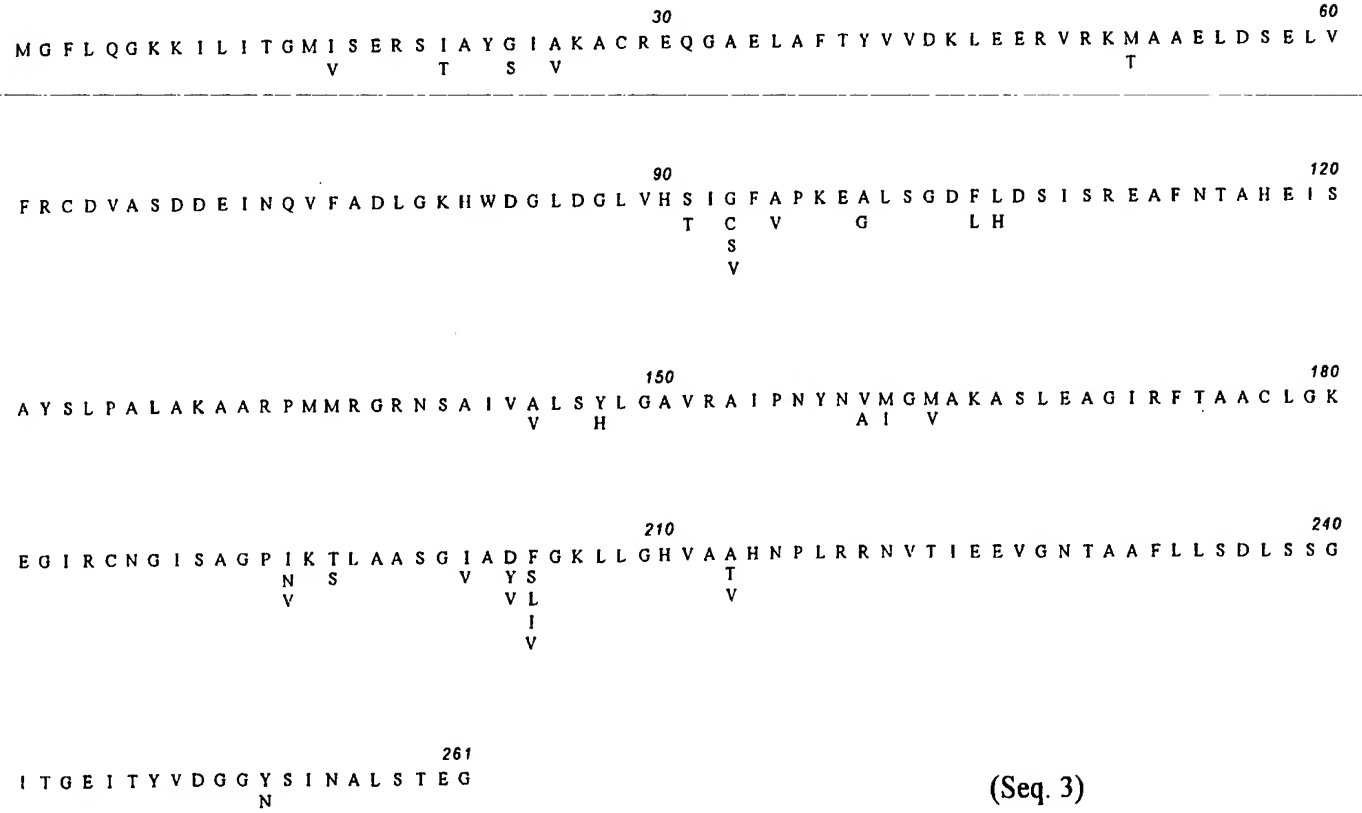
A 480 bp *gyrA* PCR product was generated using primers A (5'-GTCCGCCATGGCAGGTTTCTCGACAAAC-3') (Seq. 4) and B (5'-CATACGGACGATGGTGCCGTAAACTGCGAAATCGCCGTGGGGGTG-3') (Seq. 5) (altered restriction sites underlined). A 570 bp *gyrA* PCR product that overlaps the other *gyrA* product was generated using primers C (5'-CACCCCCACGGCGATTTCGCAGTTTACGGCACCATCGTCCGTATG-3') (Seq. 6) and D (5-CAACTTGAATTCGTTGACCTGATAGGG-3') (Seq. 7). The resulting PCR products were purified and combined with primers A and D in a PCR reaction to produce a 1050 bp fragment (called the *gyrA* SD-FG PCR product) containing the desired *gyrA* mutations.

Generation of Quinolone Resistance Determining Region Random Library

A 800 bp PCR product containing the first 600 bp of *gyrA* from *N. gonorrhoeae* was amplified using the oligonucleotides GC gyrA 5' NcoI (5'-GTCCGCCATGGCAGGTTTCTCGACAAAC -3') (Seq. 8) and GC gyrA 3' HindIII (5-**CCCAAGCTT**GATGGTGTCGGTGAGGTTG-3') (Seq. 9) (mutant residues in bold, restriction enzymes sites underlined). The resulting fragment and pAlterEX-2 (Promega) were digested with NcoI and HindIII, and ligated to create pAlt-gyrA.

To generate a pool of random insertions isolated to the *gyrA* QRDR (coding for residues 88-103), the oligonucletide GC gyrA-random (5'-cacggcgattccgcagtttacgacacAatcgtccgtatggcgcaaaatTTCGC-3') (Seq. 10) was synthesized by Integrated DNA Technologies (lower case nucleotides were synthesized using phosphoramidite stock solutions contaminated with 0.7% of each non-wild type phosphoramdite, underlined is destroyed XcmI site). The resulting pool of oligonucleotides contained an average of one random mutation per oligonucleotide. This 53-mer was used for site-specific mutagenesis of pAlt-gyrA per manufacturer's protocol (Altered Sites, Promega). To ensure all colonies resulting from the mutagenesis were not wild-type, a silent C to A mutation was generated in the primer (shown in bold) which destroyed a unique XcmI site. This allowed for all plasmids to be digested with XcmI to eliminate non-recombinant plasmids. All colonies (~4000) isolated from the mutagenesis reactions were pooled together to generate a collection of plasmids containing random single

Amino Acid Sequence of FabI and DHDPE or Triclosan Resistant Mutations



(Seq. 3)

Generation of DHDPE resistant FabI mutants

Random mutations in *fabI* were generated as described previously (Kok et al.) using the PCR product generated with Gc7 (5'-GGAATTCCATATGCGTAT TTGAAACGTCCAATGCC-3') (Seq. 13) and Gc8 (5'-GCACCTGCAGCAATGCGG TAC-3') (Seq. 14) using 10 ng N400 genomic DNA as template. PCR reactions were performed with either Taq polymerase (GIBCO-BRL) or the XL PCR kit (Perkin-Elmer). Ten independent PCR reactions were performed using each polymerase with the following reaction mixtures: 10µl 10x buffer (supplied with enzyme), 10 ng N400 genomic DNA as template, 20 pmoles primers, 200 µM dNTP, and either 1.5 mM $MgCl_2$ (for Taq) or 2.0 mM $Mg(OAc)_2$ (for XL PCR) (100µl final volume). The 20 reactions were pooled following 35 cycles of 95°C for 15 sec, 58°C for 30 seconds and 72°C for 1 minute. The resulting PCR products were ethanol precipitated and resuspended to 0.5 µg/ml in H_20 for subsequent transformation of gonococcal strains.

N400 was transformed with mutant PCR products using either the spot transformation technique on solid media or liquid transformation as described previously. The cells were than plated on GC solid media containing 0.5, 2 or 10 µg DHDPE per ml to select for DHDPE-resistant bacteria. Isolated colonies were passaged 2 times on GC solid media to ensure homogeneity. The *fabI* alleles were PCR amplified directly from colonies using Gc7 and Gc8 and sequenced. All PCR products containing *fabI* mutations were used to transform N400 and the selection process was repeated. If the frequency of obtaining resistant mutants was at least 100-times higher than when using a PCR product generated using N400 DNA as the template it was concluded that the mutation responsible for the resistant phenotype was in *fabI*.

Generation of random library via mutagenic PCR of large regions of the chromosome

PCR primers were designed using in-house software, PRIMER, in conjunction with BIGPRIME (a modification by the Genetics Computer Group of their PRIME program to allow for products up to 25 kb). PRIMER uses the BIGPRIME program to interactively design a list of oligonucleotide pairs to

generate the library of DNA fragments used in the transformation that created mutant strain. Once the mutation has been mapped to a reasonable sized portion of the chromosome, for example less than 3 kilobasepairs, using an iterative process of primer design, PCR amplification, transformation and selection of bacteria with altered susceptibility to the chemical, the DNA from the region of the mutant that carries the mutation can be sequenced. In this manner the mutation responsible for the altered susceptibility can be identified. From this the gene or genes involved in the mechanism by which the chemical affects the growth of the bacteria are identified.

This system can also be used to identify mutations in a bacterial chromosome that have been generated by other means and result in a phenotypic alteration. Examples of this are 1) strains carrying extra-chromosomal elements that result in a detectable phenotype, such as loss of virulence, fluorescence via green fluorescence protein (GFP) or resistance to an antibacterial compound; or 2) mutant strains containing point mutations that result in resistance to antibacterial compounds with known or unknown targets. In the former case, PCR products containing the entire genome can be systematically subjected to in vitro mutagenesis where any external fragment of DNA can be randomly inserted into the PCR product using the GPS system of New England Biolabs. The resulting PCR products can then be transformed into the wild-type strain, the extra-chromosomal material recombined onto the chromosome, and mutants containing the desired phenotype identified and isolated. In the latter case, resistant mutants can be generated using chemical means such as ethylenemethane sulfonate, DNA-damaging agents such as UV irradiation, or simply by isolating spontaneous mutants that grow on plates containing a concentration of the chemical compound that prevents growth of the parent strain. Once a strain carrying the detectable phenotype has been generated, PCR of the entire chromosome of the mutant organism in defined regions can be performed and the location of the mutation identified as described above.

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and the cells incubated overnight to allow for uptake and recombination of the mutant PCR products. Cells from each spot were then resuspended in 150 μL GC media and 5 μL of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used to inoculate 96-well plates containing 100 μL of GC-media supplemented with Isovitalex and an inhibitory concentration of the antibacterial agent. Following 2-4 days of incubation at 37°C with 5% CO_2, wells containing viable bacteria were streaked onto plain plates and individual colonies isolated.

Identification of Mutations Conferring Resistance

To identify the mutation responsible for the resistance phenotype, DNA from the resistant mutant was amplified in 12 independent reactions using primer pairs corresponding to the region containing the resistance mutation. These products were then used as donor DNAs in transformation experiments as described above, and the PCR product containing the resistance mutation was identified by its ability restore the resistance phenotype. By generating smaller PCR products (1-4 kb) which span the 8-12 kb PCR product conferring resistance, the transformation and selection process was repeated and the mutation mapped to a 1-2 kb fragment of DNA. The DNA sequence of this fragment was determined using fluorescence-dye sequencing on an ABI 377 and analyzed using the SEQUENCHER program (Genecodes). The resulting sequence was compared to the analogous region of wild-type DNA to identify any mutation(s).

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to His, Glu161 to Gly, Glu161 to Lys, Asn65 to His, Asp80 to Gly, and Glu62 to Lys; and

f) using these mutants to help to understand the mechanism of action of quinolones, and other type IV topoisomerase inhibitors.

3. Mutations in *Neisseria gonorrhoeae* GyrA associated with quinolone resistance selected from: Asp90 to Glu, Ser91 to Cys, Asp95 to His, Glu161 to Gly, Glu161 to Lys, Asn65 to His, Asp80 to Gly, and Glu62 to Lys.

4. The process according to Claim 1 for identifying and characterizing drug-target interactions.

5. A process for identifying and characterizing a mechanism of action of an antibacterial compound comprising:

generating DNA fragments by polymerase chain reaction amplification of DNA from bacteria under conditions that allow for mutation of the fragments;

allowing one or more of the generated DNA fragments to be incorporated into the chromosome of a bacteria by homologous recombination;

isolating the bacteria that demonstrate resistance to an antibacterial compound; and

identifying the mutation contained in the DNA fragment.

6. A process for identifying mutations contained in the chromosome of a bacteria that results in an identifiable phenotype comprising:

a) generating DNA fragments by polymerase chain reaction amplification of the bacterial chromosome corresponding to regions of the bacterial chromosome which may contain a mutation;

b) allowing one or more of the DNA fragments to be incorporated into the chromosome of a bacteria that does not display the identifiable phenotype by homologous recombination;

Generation of DHDPE resistant FabI mutants

Random mutations in *fabI* were generated as described previously (Kok et al.) using the PCR product generated with Gc7 (5'-GGAATTCCATATGCGTAT TTGAAACGTCCAATGCC-3') (Seq. 13) and Gc8 (5'-GCACCTGCAGCAATGCGG TAC-3') (Seq. 14) using 10 ng N400 genomic DNA as template. PCR reactions were performed with either Taq polymerase (GIBCO-BRL) or the XL PCR kit (Perkin-Elmer). Ten independent PCR reactions were performed using each polymerase with the following reaction mixtures: 10µl 10x buffer (supplied with enzyme), 10 ng N400 genomic DNA as template, 20 pmoles primers, 200 µM dNTP, and either 1.5 mM $MgCl_2$ (for Taq) or 2.0 mM $Mg(OAc)_2$ (for XL PCR) (100µl final volume). The 20 reactions were pooled following 35 cycles of 95°C for 15 sec, 58°C for 30 seconds and 72°C for 1 minute. The resulting PCR products were ethanol precipitated and resuspended to 0.5 µg/ml in H_20 for subsequent transformation of gonococcal strains.

N400 was transformed with mutant PCR products using either the spot transformation technique on solid media or liquid transformation as described previously. The cells were than plated on GC solid media containing 0.5, 2 or 10 µg DHDPE per ml to select for DHDPE-resistant bacteria. Isolated colonies were passaged 2 times on GC solid media to ensure homogeneity. The *fabI* alleles were PCR amplified directly from colonies using Gc7 and Gc8 and sequenced. All PCR products containing *fabI* mutations were used to transform N400 and the selection process was repeated. If the frequency of obtaining resistant mutants was at least 100-times higher than when using a PCR product generated using N400 DNA as the template it was concluded that the mutation responsible for the resistant phenotype was in *fabI*.

Generation of random library via mutagenic PCR of large regions of the chromosome

PCR primers were designed using in-house software, PRIMER, in conjunction with BIGPRIME (a modification by the Genetics Computer Group of their PRIME program to allow for products up to 25 kb). PRIMER uses the BIGPRIME program to interactively design a list of oligonucleotide pairs to

16. The process of claims 1 or 2 in which the antibacterial compound inhibits the growth or survival of the bacteria under any condition.

17. The process of claims 1 or 2 in which the antibacterial compound inhibits the growth or survival of the bacteria in culture.

18. The process of claims 1 or 2 in which the antibacterial compound inhibits the growth or survival of the bacteria in an animal host.

19. The process of claims 1 or 2 in which the antibacterial compound is an inhibitor of type II topoisomerases.

20. The process of claims 1 or 2 in which the antibacterial compound is an inhibitor of FabI.

21. The process of claims 1 or 2 in which the antibacterial compound is an inhibitor of enzymes involved in fatty acid biosynthesis.

22. The process of claim 6 in which a strain of bacteria carrying the mutation was isolated from a culture that had been treated with a chemical mutagen.

23. The process of claim 6 in which a strain of bacteria carrying the mutation was isolated from a culture that had been treated with ultraviolet light.

24. The process of claim 6 in which a strain of bacteria carrying the mutation was isolated from a culture in which the bacteria had been subjected to a mutagenic protocol that consisted of insertion of DNA into the chromosome of the bacteria.

25. Bacteria comprising a protein in which a continguous stretch of 40 amino acids is at least 30% identical to residues 75 to 114 of the *Neisseria gonorrrhoeae* GyrA and the residue analogous to:
62 is lysine or

and the cells incubated overnight to allow for uptake and recombination of the mutant PCR products. Cells from each spot were then resuspended in 150 μL GC media and 5 μL of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used to inoculate 96-well plates containing 100 μL of GC-media supplemented with Isovitalex and an inhibitory concentration of the antibacterial agent. Following 2-4 days of incubation at 37°C with 5% CO_2, wells containing viable bacteria were streaked onto plain plates and individual colonies isolated.

Identification of Mutations Conferring Resistance

To identify the mutation responsible for the resistance phenotype, DNA from the resistant mutant was amplified in 12 independent reactions using primer pairs corresponding to the region containing the resistance mutation. These products were then used as donor DNAs in transformation experiments as described above, and the PCR product containing the resistance mutation was identified by its ability restore the resistance phenotype. By generating smaller PCR products (1-4 kb) which span the 8-12 kb PCR product conferring resistance, the transformation and selection process was repeated and the mutation mapped to a 1-2 kb fragment of DNA. The DNA sequence of this fragment was determined using fluorescence-dye sequencing on an ABI 377 and analyzed using the SEQUENCHER program (Genecodes). The resulting sequence was compared to the analogous region of wild-type DNA to identify any mutation(s).

29. A protein comprising in which a contiguous stretch of 40 amino acids is at least 30% identical to residues 75 to 114 of the *Neisseria gonorrrhoeae* GyrA and the residue analogous to:
62 is lysine or
63 is arginine or glutamic acid or
65 is histidine or
135 is valine or
161 is glutamic acid or lysine or glycine.

30. *Neisseria gonorrheae* GyrA protein comprising amino acid substitutions when residue
62 is lysine, or
63 is arginine or glutamic acid, or
65 is histidine, or
80 is alanine or glycine, or
90 is arginine or glutamic acid, or
91 is tyrosine or alanine or cysteine, or
92 is proline, or
95 is arginine or alanine or valine or tyrosine or histidine or glycine, or
114 is histidine, or
135 is valine, or
161 is glutamic acid or lysine or glycine.

31. Bacteria comprising a protein that is at least 30% identical to the sequence of the *Neisseria gonorrhoeae* FabI protein in which the amino acid residue corresponding to
15 is valine, or
20 is threonine, or
23 is glycine, or
25 is valine, or
51 is threonine, or
91 is threonine, or
93 is cysteine or serine, or

to His, Glu161 to Gly, Glu161 to Lys, Asn65 to His, Asp80 to Gly, and Glu62 to Lys; and

f) using these mutants to help to understand the mechanism of action of quinolones, and other type IV topoisomerase inhibitors.

3. Mutations in *Neisseria gonorrhoeae* GyrA associated with quinolone resistance selected from: Asp90 to Glu, Ser91 to Cys, Asp95 to His, Glu161 to Gly, Glu161 to Lys, Asn65 to His, Asp80 to Gly, and Glu62 to Lys.

4. The process according to Claim 1 for identifying and characterizing drug-target interactions.

5. A process for identifying and characterizing a mechanism of action of an antibacterial compound comprising:

generating DNA fragments by polymerase chain reaction amplification of DNA from bacteria under conditions that allow for mutation of the fragments;

allowing one or more of the generated DNA fragments to be incorporated into the chromosome of a bacteria by homologous recombination;

isolating the bacteria that demonstrate resistance to an antibacterial compound; and

identifying the mutation contained in the DNA fragment.

6. A process for identifying mutations contained in the chromosome of a bacteria that results in an identifiable phenotype comprising:

a) generating DNA fragments by polymerase chain reaction amplification of the bacterial chromosome corresponding to regions of the bacterial chromosome which may contain a mutation;

b) allowing one or more of the DNA fragments to be incorporated into the chromosome of a bacteria that does not display the identifiable phenotype by homologous recombination;

23 is glycine, or
25 is valine, or
51 is threonine, or
91 is threonine, or
93 is cysteine or serine, or
95 is valine, or
104 is leucine, or
105 is histidine, or
144 is valine, or
147 is histidine, or
159 is alanine, or
160 is isoleucine, or
162 is valine, or
193 is asparagine or valine, or
201 is valine, or
203 is tyrosine or valine, or
204 is serine or leucine or isoleucine or valine, or
212 is threonine or valine, or
247 is asparagine.

35. A *Neisseria gonorrhoeae* FabI protein comprising the amino acid corresponding to residue:
15 is valine, or
20 is threonine, or
23 is glycine, or
25 is valine, or
51 is threonine, or
91 is threonine, or
93 is cysteine or serine, or
95 is valine, or
104 is leucine, or
105 is histidine, or
144 is valine, or

16. The process of claims 1 or 2 in which the antibacterial compound inhibits the growth or survival of the bacteria under any condition.

17. The process of claims 1 or 2 in which the antibacterial compound inhibits the growth or survival of the bacteria in culture.

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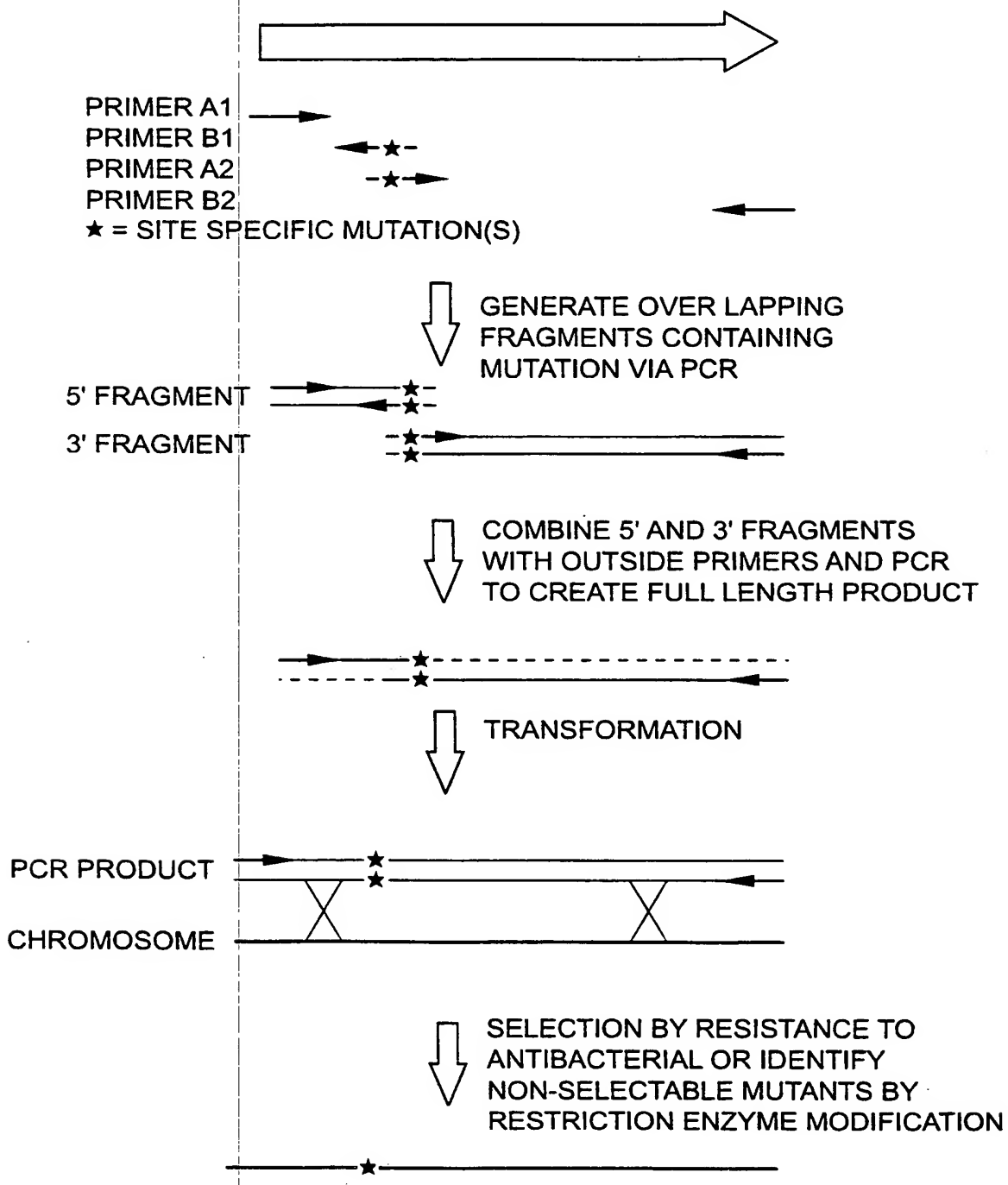
62 is lysine or

FIG-1 RAPID GENERATION OF SITE-SPECIFIC CHROMOSOMAL MUTANTS



29. A protein comprising in which a contiguous stretch of 40 amino acids is at least 30% identical to residues 75 to 114 of the *Neisseria gonorrrhoeae* GyrA and the residue analogous to:
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63 is arginine or glutamic acid or
65 is histidine or
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30. *Neisseria gonorrheae* GyrA protein comprising amino acid substitutions when residue
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80 is alanine or glycine, or
90 is arginine or glutamic acid, or
91 is tyrosine or alanine or cysteine, or
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31. Bacteria comprising a protein that is at least 30% identical to the sequence of the *Neisseria gonorrhoeae* FabI protein in which the amino acid residue corresponding to
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23 is glycine, or
25 is valine, or
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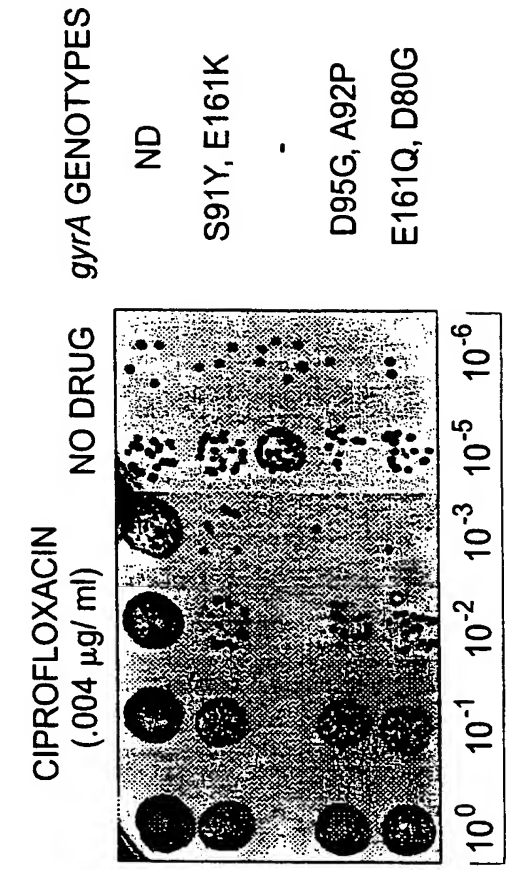
91 is threonine, or
93 is cysteine or serine, or

FIG-3



23 is glycine, or
25 is valine, or
51 is threonine, or
91 is threonine, or
93 is cysteine or serine, or
95 is valine, or
104 is leucine, or
105 is histidine, or
144 is valine, or
147 is histidine, or
159 is alanine, or
160 is isoleucine, or
162 is valine, or
193 is asparagine or valine, or
201 is valine, or
203 is tyrosine or valine, or
204 is serine or leucine or isoleucine or valine, or
212 is threonine or valine, or
247 is asparagine.

35. A *Neisseria gonorrhoeae* FabI protein comprising the amino acid corresponding to residue:
15 is valine, or
20 is threonine, or
23 is glycine, or
25 is valine, or
51 is threonine, or
91 is threonine, or
93 is cysteine or serine, or
95 is valine, or
104 is leucine, or
105 is histidine, or
144 is valine, or

SEQUENCE LISTING

<110> Dunham, Steven
Olson, Eric

<120> A MODEL SYSTEM FOR DRUG DELIVERY

<130> PR.3021.001 60/105,965 DRUG DISCOVERY

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FIG-1 RAPID GENERATION OF SITE-SPECIFIC CHROMOSOMAL MUTANTS

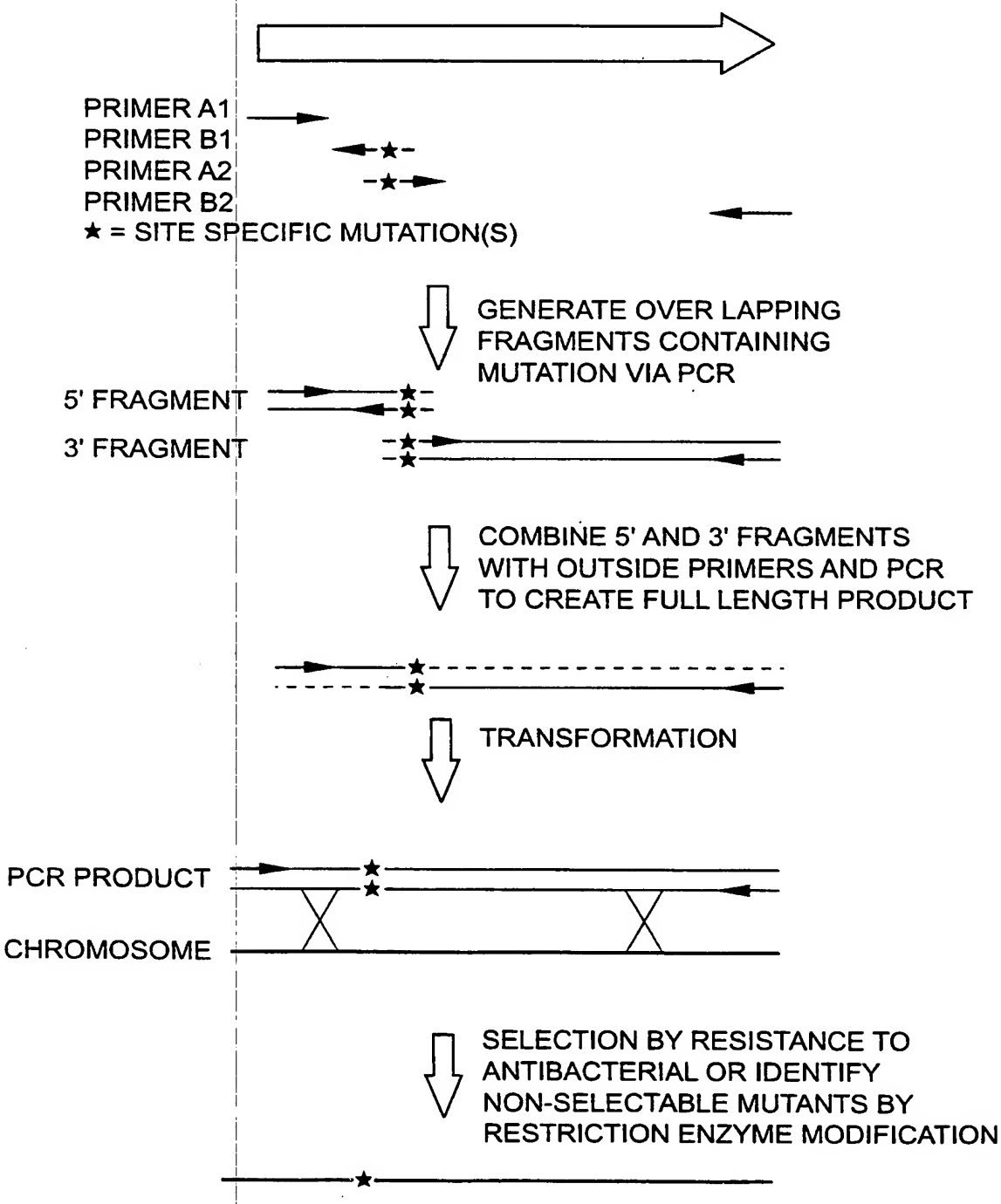

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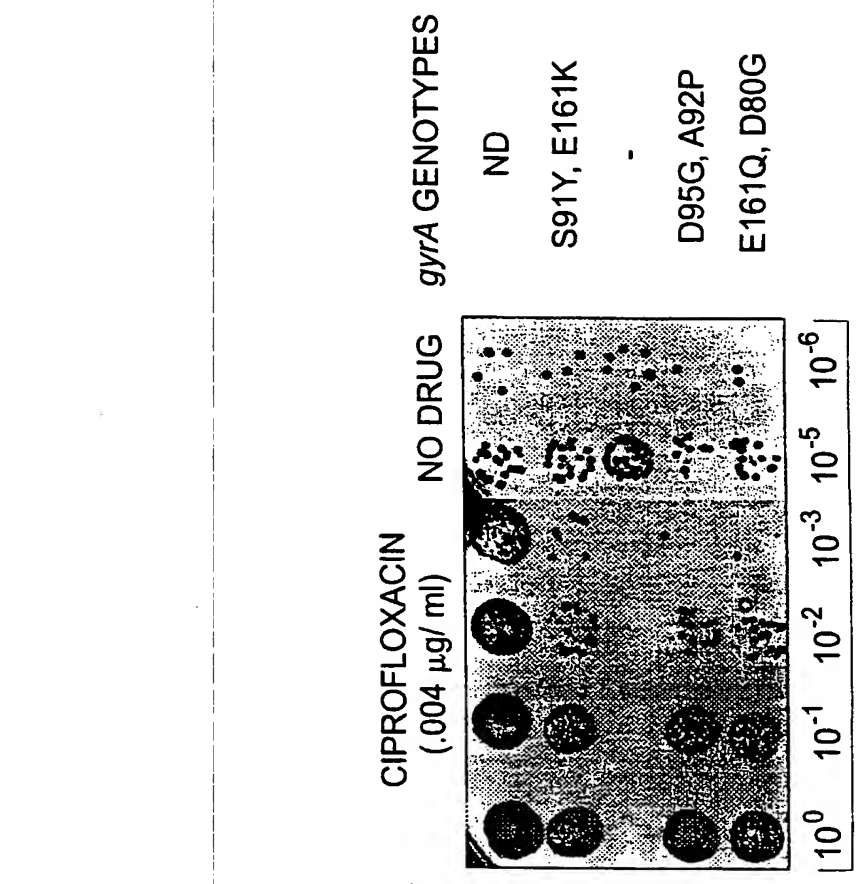
FIG-3



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Glu Val Gly Asn Thr Ala Ala Phe Leu Leu Ser Asp Leu Ser
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SEQUENCE LISTING

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Olson, Eric

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<130> PR.3021.001 60/105,965 DRUG DISCOVERY

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 99/22118

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07K14/22 C07K14/245 C12R1/19 C12R1/36
C12N15/10 C12Q1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEGUCHI T ET AL.: "Quinolone-resistant Neisseria gonorrhoeae: Correlation of alterations in the gyrA subunit of DNA gyrase and the parC subunit of topoisomerase IV with antimicrobial susceptibility profiles" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 40, no. 4, 1996, pages 1020-1023, XP000870143 cited in the application	27,28, 30,32
Y	* see especially table 1 and fig. 1 * the whole document	1,2,4-6, 8-11,15, 22,36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Knehr, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MCMURRY L M ET AL: "Triclosan targets lipid synthesis" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 394, no. 394, 6 August 1998 (1998-08-06), pages 531-532-532, XP002108567 ISSN: 0028-0836 cited in the application the whole document	5,6,8,9, 14,15, 20-22, 24,36
A	DEGUCHI T ET AL.: "DNA gyrase mutations in quinolone-resistant clinical isolates of Neisseria gonorrhoeae" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 39, no. 2, 1995, pages 561-563, XP000870151 cited in the application the whole document	
A	WEIGEL L ET AL: "GyrA mutations associated with fluoroquinolone resistance in eight species of enterobacteriaceae" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, vol. 42, no. 10, October 1998 (1998-10), pages 2661-2667-67, XP002118443 ISSN: 0066-4804 the whole document	
A	EP 0 688 873 A (BAYER AG) 27 December 1995 (1995-12-27) the whole document	
P,X	HEATH R J: "Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 273, no. 46, 13 November 1998 (1998-11-13), pages 30316-30320-30320, XP002108571 ISSN: 0021-9258	31,34
P,Y	* see especially page 30318, column 2, paragraph 2 * the whole document	5,6,9, 13-15, 20,35

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21

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 99/22118

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 8000298 A	09-01-1996

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

1

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